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Resistance of *Bacillus subtilis* var. *niger* Spores Occluded in Water-insoluble Crystals to Three Sterilization Agents

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The resistance to destruction of spores of *Bacillus subtilis* var. *niger* occluded in crystals of calcium carbonate and exposed to ethylene oxide and moist and dry heat was determined and compared with the destruction of unoccluded spores. Occluded spores could not be inactivated with ethylene oxide. Resistance to inactivation was approximately 900 and 9 times higher for occluded than for unoccluded spores subjected to moist and dry heat, respectively, at 121 C. The protective effect may be due either to the unavailability of oxygen for destruction by oxidation or to inhibition of the loss of essential cell constituents by vaporization. Evidence also implicates the crystal structure as a thermal conductivity barrier. Occluded spores retained viability over a 3-year period compared with unoccluded spores which decreased over 90% during this period. Occluded spores in insoluble materials are seldom encountered in the technology of sterilization, but could be the most critical factor in the sterilization of interplanetary vehicles. Entrapped spores in insoluble materials are usually difficult to detect, and are very stable as well as extremely resistant to destruction by heat and ethylene oxide.

Many papers have been written on factors which influence the resistance of microorganisms to a sterilizing agent. Sobernheim and Mündel (13) found that spores in soil were six to eight times more resistant to sterilization by moist heat than the same number of spores in a laboratory culture. We have found soils having a much higher resistance to dry heat, moist heat, and ethylene oxide than spore isolates cultured from the same soil.

The occlusion and survival of microorganisms in crystals have been reported previously. Abbot, Cockton, and Jones (1), using various water-soluble crystals, observed an increased resistance to destruction in formaldehyde and ethylene oxide. They demonstrated the inclusion of organisms in crystals by use of the electron microscope. Royce and Bowler (10) also noted that protection from gaseous ethylene oxide was provided by water-soluble crystals.

Bacteria were seen in crystals of calcite more than 100 million years old by Bradley (4). Ehlers, Stiles, and Birle (7) and Schopf et al. (11) have reported iron bacteria in pyrite. Barghoorn and Schopf (2) and Barghoorn and Tyler (3) have also reported on microorganisms present in geological formations. No attempt was made by the preceding investigators to see whether the microorganisms were viable.

Apparently no one has investigated the viability or resistance of microorganisms in water-insoluble crystals. If a crystal is grown very rapidly so that impurities, such as microorganisms, do not have time to be desorbed, they may be entrapped within the growing crystal. Recovery would be impossible with ordinary culturing techniques, since they would not be released from the crystalline matrix. For this study, calcium carbonate was chosen because it is relatively insoluble in water and easily dissolved in dilute acid or ammonium chloride. Other insoluble crystalline materials were also studied.

MATERIALS AND METHODS

Organism. Bacillus subtilis var *niger* 356 S.C. no. 4 N. R. Smith strain was used (12). This strain has a relatively high dry-heat and ethylene oxide resistance, but relatively low moist-heat resistance.

Culture medium. Spores of the test organism were heat-shocked at 65 C for 30 min and were used as inocula for starter cultures in casein acid digest medium of the following composition: casein acid hydrolysate powder (General Biochemicals, Inc., Chagrin Falls, Ohio), 10.0 g; yeast extract (Difco), 5.0 g; glucose, 5.0 g; KH_2PO_4, 5.0 g; $CaCl_2 \cdot 2H_2O$, 0.66 g; $MnSO_4 \cdot H_2O$, 0.03 g; tap water, 1,000 ml. The

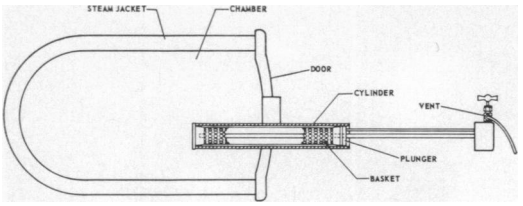

FIG. 1. *Diagrammatic representation of the autoclave used for determination of moist-heat parameters.*

medium was filtered, adjusted to *p*H 7.0, and sterilized in steam at 121 C for 20 min.

After shaking for 24 hr at 32 C, the starter culture was used to inoculate large flasks of the same medium. These were incubated with shaking for 4 days at 32 C, and the sporulated culture was kept at 45 C overnight to allow autolysis of vegetative cells. The spores were then harvested by centrifugation, washed eight times in distilled water, and checked by phase microscopy to ascertain absence of debris.

Preparation of occluded spores. A 10-ml solution of 1.11% $CaCl_2$ was prepared containing 10^8 spores/ml. To this solution, 10 ml of 1.06% Na_2CO_3 was added very rapidly and the mixture was vigorously shaken. Crystals of Ca_2CO_3 were immediately formed, occluding large quantities of spores per crystal. The crystals were then centrifuged at 20,500 $\times$ *g*, washed three times with distilled water, and made up to original volume. Methocel (0.2 g, 25 centipoises/sec; Dow Chemical Co., Midland, Mich.) was added to the crystal suspension to make the solution viscous enough to suspend the crystals evenly, and to bind the crystals to the paper on which they were inoculated. Samples of 0.01 ml were used to inoculate the paper strips. The strips were first dried at room temperature, then at 90 C for 16 hr to remove any occluded water. When the strips were not air-dried at 90 C, the resistance of the spores to moist or dry heat did not increase, though it did for ethylene oxide.

To verify that the spores were occluded and not adsorbed to the crystals, the crystallization procedure was followed by use of sterile solutions. Then, after crystallization, spores were added and the procedure was followed as before. Strips made with this procedure had no increased moist-heat, dry-heat, or ethylene oxide resistance. Microscopic examination showed free spores among the crystals.

Recovery of occluded spores for assaying and sterility end-point determination. After exposure to the sterilizing agent, the spore strips were placed in 25 ml of sterile 3% NH_4Cl for 3 days at 0 C to dissolve the $CaCO_3$. The solution and strip were then placed in a Waring Blendor at high speed for 3 min. The solution was then sonic-treated at 21 kc for 5 min to eliminate clumping. Appropriate dilutions were made on tryptone-glucose-yeast extract-agar (Difco) and incubated at 37 C for 48 hr before counting. This technique did not affect the counts on the unoccluded spore strip controls. For sterility end-point determinations, the above procedure was followed except that the spore strips and NH_4Cl solution were added to 25 ml of double-strength Trypticase Soy Broth (BBL) after the crystals were dissolved.

Determination of resistance to ethylene oxide. Apparatus and procedures similar to those reported by Ernst and Shull (7) were used.

Determination of resistance to moist heat. An autoclave, 9 inches inside diameter and 16 inches deep (Fig. 1), was used for determining moist-heat resistance. A similar device was described by Cook and Brown (6). Initially, the large chamber was evacuated, after which steam was added to the desired pressure and temperature. The test spore strips were placed in the plunger chamber which was then evacuated. The plunger was pushed into the steam chamber, thus immediately exposing the spore strips to the saturated steam at the desired temperature. After a predetermined time interval, the plunger was pulled out of the steam chamber and the pressure and temperature were reduced instantaneously.

Determination of resistance to dry heat. A cylindrical aluminum block, 4 inches in diameter and 6⅜ inches in height (Fig. 2), was used for determining dry-heat resistance, and was similar to that used by Bruch, Koesterer, and Bruch (5). It was equipped with a central heating element and a temperature regulator. The temperature was uniform throughout the entire tube and in the six replicate tubes and could be monitored routinely with thermocouples or thermom-

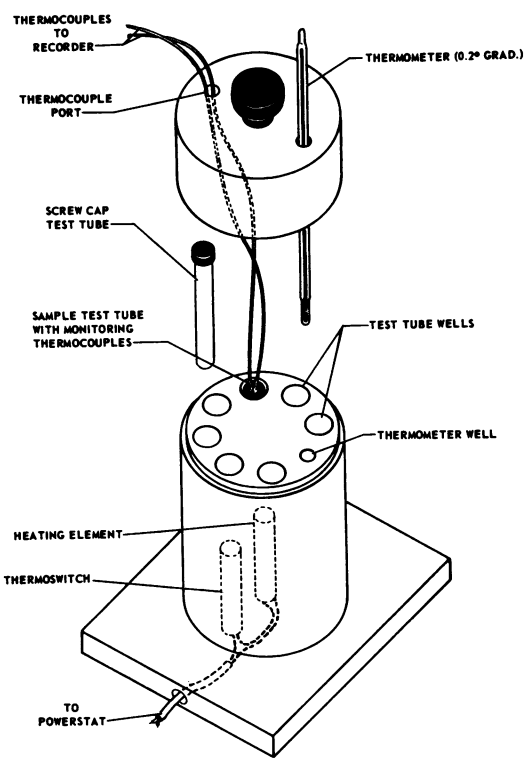

FIG. 2. *Diagrammatic representation of the aluminum block unit used for determination of dry-heat parameters.*

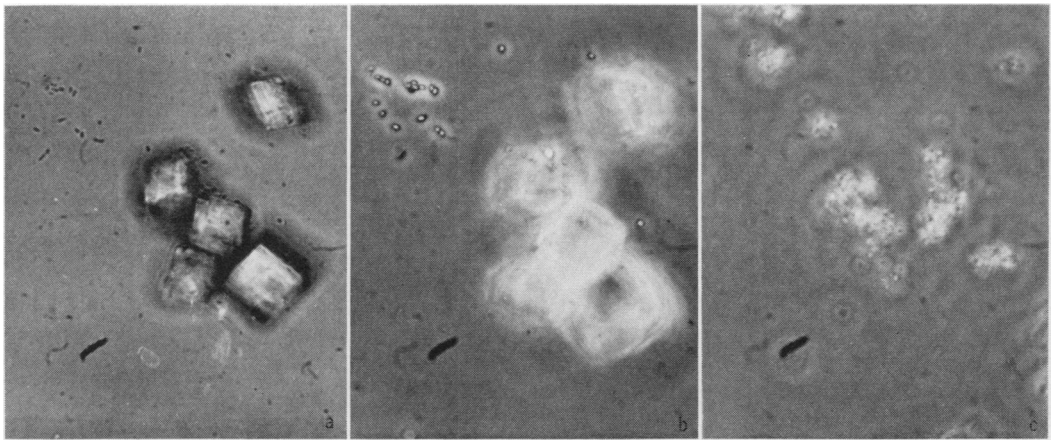

FIG. 3. *Photomicrographs of crystals and various stages of dissolution.* × *970. (a) Crystals prior to addition of 0.1 N HCl. (B) Crystals in the process of dissolving, approximately 1 min after addition of 0.1 N HCl. (C) Clumped spores that are left after the crystals are dissolved, approximately 5 min after addition of 0.1 N HCl.*

eters (or both). When the tubes were placed in the unit, the temperature came up in less than 1 min. After the predetermined exposure time, the tubes were removed from the unit and submerged in ice water.

RESULTS

Microscopic examination. A loopfull of a water suspension of crystals containing spores was placed on a slide and air-dried. A thin film of 1.5% agar was placed over the crystals and allowed to harden, and a cover slip was placed over the agar. A drop of 0.1 N HCl was added to the edge of the agar. Shortly thereafter, the acid diffused into the crystals, causing them to dissolve slowly. A large number of spores was occluded in a single crystal (Fig. 3).

Ethylene oxide resistance. Figure 4 compares the ethylene oxide resistance of unoccluded versus occluded spores of *B. subtilis* var. *niger*. At 54 C, 40% relative humidity (RH), and with 1,200 mg of ethylene oxide per liter, 8×10^3 spores were killed in 30 sec when they were not occluded. The initial drop in count on the strips containing occluded spores was probably due to the killing of unoccluded spores present. Continued exposure to ethylene oxide for 2 weeks did not reduce the count beyond that of the first 5 sec. Of a population of 8×10^3 spores, 25%, or 2×10^3 spores, were not killed and, hence, may have been occluded. Since this method probably killed the unoccluded spores, the strips for the moist- and dry-heat experiments were first treated at 54 C, 40% RH, with 1,200 mg of ethylene oxide for 1 hr.

Moist-heat resistance. Figure 5 compares the resistance of unoccluded and occluded spores of *B. subtilis* var. *niger* to saturated steam at 121 C. The unoccluded spores were killed so quickly in

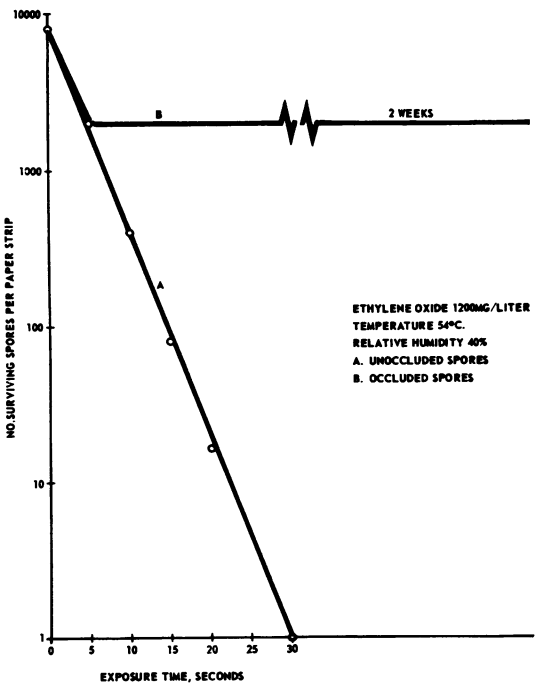

FIG. 4. *Survivor curves for unoccluded and occluded spores of Bacillus subtilis var. niger exposed to ethylene oxide.*

saturated steam that the curve was drawn based on the starting number and sterility end point. The occluded spores, on the other hand, were quite resistant to moist heat. After 1 hr, an average of less than 10 spores per paper strip was evident. This low number of spores required an additional 1.5 hr of exposure before inactivation was attained.

It was observed that occluded spores are about 900 times more resistant than unoccluded spores to moist heat at 121 C.

Dry-heat resistance. Figure 6 compares the resistance of unoccluded and occluded spores of *B. subtilis* var. *niger* to dry heat at 121 C. This temperature was chosen so that steam resistance could be compared with dry-heat resistance. The unoccluded spores were killed in a logarithmic manner in 5.5 hr. When the mortality curve for the occluded spores was extrapolated to zero-time, a lower apparent starting number, 8.9×10^2 spores, was obtained. This we believe represents spores completely protected from dry heat. The remaining 1.1×10^3 spores are partially protected. The latter were perhaps not tightly surrounded with the crystalline matrix. However, these were incapable of being inactivated with ethylene oxide as stated previously.

Occluded spores in crystals were at least nine times more resistant than unoccluded spores to dry heat at 121 C. Moist heat was more effective than dry heat on occluded spores, but occlusion of spores retarded inactivation by steam much more severely than inactivation by dry heat. However, saturated steam was a much faster sterilizing agent than dry heat whether the spores were occluded or not.

Viability of spores in crystals. An interesting sidelight of these experiments is the fact that the occluded spores showed no loss of viability or drop in count after 3 years of storage at room temperature. Washed spores of this strain of *B. subtilis* on paper tended to decrease in number with time. After 3 years, one can expect at least 2 logs decrease in count. It is not unusual for spores to survive long periods in soil. However, we believe that maintaining viability of all the cells of a particular population is unusual.

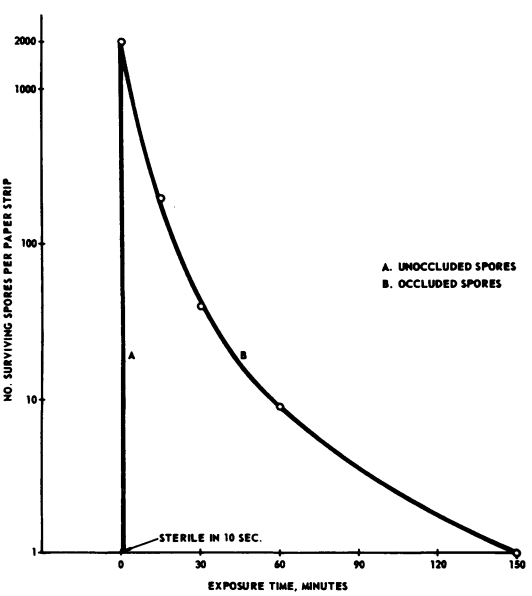

FIG. 5. *Survivor curves for unoccluded and occluded spores of Bacillus subtilis var. niger exposed to saturated steam at 121 C.*

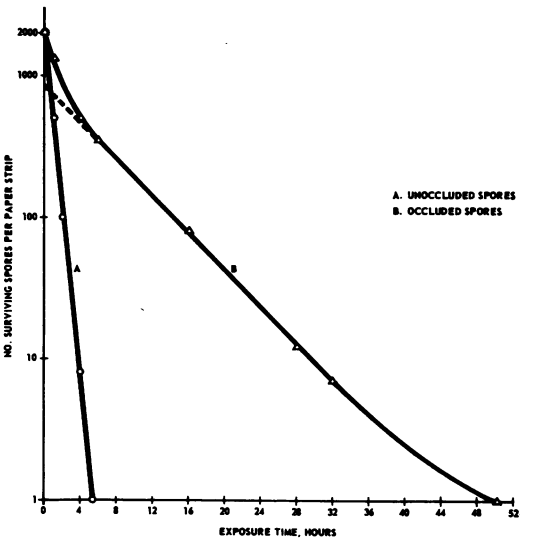

FIG. 6. *Survivor curves for unoccluded and occluded spores of Bacillus subtilis var. niger exposed to dry heat at 121 C.*

DISCUSSION

It has been shown that ethylene oxide cannot inactivate spores entrapped in water-insoluble crystals. Spores occluded in water-soluble crystals are also difficult to inactivate with ethylene oxide. However, they may be killed when the crystals are dissolved in water or when the partial pressure of the relative humidity around the crystals exceeds the vapor pressure of a saturated solution of the crystals. This is because the crystals would be continually dissolving and reforming in such a system. Ethylene oxide treatment of a population may provide a good tool for the determination of the percentage of a spore population which is occluded.

There are two possibilities why moist heat is still more effective than dry heat on occluded spores. (i) Because there is more energy per molecule of saturated steam than there is per molecule of dry air at any given temperature, steam provides a greater heat transfer through the crystals. (ii) The crystalline matrix impedes the diffusion of steam to the spores, and only when steam saturates the spore do we get killing with moist heat. Prior to saturation, killing would be primarily due to dry heat or a combination of moist and dry heat.

There are three possibilities why dry heat takes so much longer to destroy occluded spores compared with unoccluded spores. First, if the nature of dry-heat sterilization is due to oxidation, the crystalline material may impede the diffusion of oxygen to the cell, thus extending the time to sterilize. The difficulty with this idea is that inert gases have been reported to be slightly more effective than air or oxygen (9), and nitrogen to be equal to air (J. A. Rowe and M. G. Koesterer, Bacteriol. Proc., p. 8, 1965) on the rate of destruction of spores by dry heat.

A second possibility is that dry-heat sterilization is due in part to vaporization of essential cell components. The crystalline matrix could impede this diffusion, thus extending the time to sterilize. An analogous situation exists in the diffusion of gases through plastic films. For example, nylon has a high permeability to water vapor. If polyethylene is laminated to the nylon, its water vapor transmission rate is decreased because polyethylene has a low water vapor permeability.

However, the most likely explanation is that heat is transferred poorly within the crystal. This possibility is supported by the observation in our laboratory that flowing air at 121 C sterilizes in about 50% of the time required by static air.

The phenomenon of protection to a sterilizing agent and viability of spores occluded in water-insoluble crystals perhaps has not been observed before because ordinary culturing techniques would not dissolve such crystals, thereby preventing outgrowth of the organisms. We have observed similar protection and viability with other water-insoluble compounds such as calcium alginate, dimethylglyoxime, zinc carbonate, barium carbonate, and 1-aminoanthraquinone. This phenomenon is expected to be prevalent in nature, but is difficult to prove since the procedures required to recover such organisms may well enhance their destruction as well.

We (*unpublished data*) have attempted to recover known inocula of spores from materials such as wax, vaseline, and mineral oil by solvent extraction, followed by membrane filtration to retain the organisms and subsequent culturing or assaying of the filter. Some solvents, such as carbon tetrachloride, benzene, and petroleum ether, were deleterious to the spores and, consequently, were inadequate for recovering them. However, other solvents, such as acetone and trichlorotrifluoroethane, were not and the procedure was adequate for recovering a known inoculum of spores.

The fact that spores can exist within crystals and maintain their viability for a long period of time may not present a problem to the field of sterilization generally, because the spores would not ordinarily be released to a nutrient. There are, however, some implications in the field of spacecraft sterilization. Low numbers of spores entrapped within relatively impermeable material may also exhibit abnormally high resistance to dry heat. It may be difficult to assure sterility of a spacecraft because the viability of occluded spores is so difficult to determine.

ACKNOWLEDGMENT

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